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Spina bifida and folate-related genes: A study of gene–gene interactions

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Purpose: To assess whether interactions of common alleles of two folate genes contribute to spina bifida risk. **Methods:** Case-control study, comparing 203 children with spina bifida to 583 controls. **Results:** Homozygosity for the 677C-T allele of 5,10-methylenetetrahydrofolate reductase (MTHFR) alone was associated with an odds ratio for spina bifida of 1.57 (95% confidence interval [CI], 1.02–2.38). For the 844ins68 allele of cystathionine-β-synthase alone, the odds ratio was 0.83 (95% CI, 0.39–1.64). For the joint genotype, the odds ratio was 3.69 (95% CI, 1.04–13.50). **Conclusions:** Interactions between common alleles of folate genes might contribute to the risk for spina bifida. ***Genet Med* 2002:4(3):126–130.**

Key Words: *spina bifida, folate, genetics, epidemiology, interaction*

Spina bifida is a common and severe congenital anomaly that yearly affects approximately 1 in 1,000 newborns in Italy[1] and 200,000 newborns or more worldwide.[2,3] Clinical and epidemiologic evidence, such as the variability of occurrence rates by geography, time, socioeconomic status, and ethnicity, and the relatively low recurrence risk within families, suggests that the etiology of spina bifida involves interactions between multiple genetic and environmental factors.[4,5] However, defining these factors and interactions has proven difficult. The protective effect of folic acid on the occurrence of spina bifida[6–8] suggests that the study of folate metabolism might identify some of the genetic determinants of spina bifida. Several candidate genes have been studied, including 5,10-methylenetetrahydrofolate reductase (MTHFR), cystathionine-β-synthase (CBS), methionine synthase, and methionine synthase reductase. A pooled analysis suggests that homozygosity for the 677C-T allele of MTHFR in infants is associated with approximately a 70% increased risk for spina bifida.[9] Common mutations of other genes, such as the 844ins68 allele of CBS,[10] have also been described, although their association with spina bifida is still unclear. Limited data are available on potential interactions among alleles of different folate-related genes. In 1997, Ramsbottom and associates[11] from Ireland reported data on the 677C-T allele of MTHFR and the 844ins68 allele of CBS and suggested that they indicated no interaction effects for spina bifida. We suggested that these data were consistent with the presence of interaction,[12] although the original authors thought otherwise.[13] Subsequent studies yielded discordant findings, with one study from Germany reporting no interaction effects[14] and another study from the United States reporting possible evidence for interaction.[15] In this report, which expands findings reported in abstract,[16] we assess the independent and joint effects of these two common alleles (677C-T and 844ins84) on the risk for spina bifida, using data from a large case-control study from Italy.

MATERIALS AND METHODS

Study population

The study population comprised 203 children with open spina bifida without other unrelated major malformations (case-subjects) and 583 healthy young adults and newborns (control-subjects). The subjects with spina bifida had been included in another study of MTHFR allelic variants.[17] Of the 203 children with spina bifida, 173 (85%) had myelomeningocele and 30 (15%) had lipomeningocele. All affected children were enrolled from three spina bifida centers in three cities (Genova, Roma, Napoli) with the assistance of the Italian Federation of Spina Bifida and the Hydrocephalus Association. All children were born in Italy from Italian mothers. Children born in Sardinia were excluded. All affected children were alive at the time of the study, and their ages ranged from 1 month to 7 years. All had no affected sibs.

Of the 583 control subjects, 306 (52%) were healthy young adults who had contributed samples to an anonymous DNA bank. Their ages ranged from 20 to 49 years. Some were healthy volunteers, others were parents of children with genetic diseases. None had a first-degree relative with a neural tube defect. The other 277 control subjects (48%) were newborns whose blood spots were obtained from the regional newborn screening programs serving the areas where the case-subjects were born. The samples from all control subjects were anonymous,

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and information associated with these samples (newborns and adults alike) included sex, region of birth, and age group.

Genetic assessment

We assessed the subjects' genotype relative to the 677C-T allele of MTHFR and the 844ins68 allele of CBS. DNA was prepared from blood following standard procedures. For blood spots, DNA was eluted from the paper (30 minutes at 96°C in 130 μL of distilled water covered with a drop of paraffin oil) and was directly used for polymerase chain reaction amplification by adding the amplification mix to the template. The 677C-T allele and the 844ins68 alleles were detected as previously reported.[18,19]

Estimating risks and interactions

To describe the independent and joint effects of the gene variants, we used the "two-by-four table" approach discussed in relation to gene-environment[19–21] and gene–gene interaction.[12,19] We classified subjects in four mutually exclusive groups, by whether or not they were homozygous for the 677C-T allele and whether or not they had the 844ins68 allele (Table 1). The reference category was that in which neither the 677C-T allele nor the 844ins68 allele was present. For the other three genotypic groups, we computed odds ratios, both unadjusted and adjusted for birth region and sex. We present unadjusted odds ratios with Fisher-exact confidence intervals (SABER, L. James, Centers for Disease Control and Prevention). We computed the attributable fraction among those with the genotype of interest (AF-gen), as

$$\text{AF-gen} = (\text{OR} - 1)/\text{OR}$$

which is analogous to the attributable fraction among the exposed in classic epidemiology, and the attributable fraction in the population (AF-pop), as

$$\text{AF-pop} = [f_{ca}(\text{OR} - 1)]/\text{OR}$$

where f_{Ca} is the fraction of cases with the genotype under study and OR is the odds ratio.[22] We used the SAS statistical package (version 6.12, SAS Institute, Inc., Cary, NC) to generate log-linear models (CATMOD procedure) that tested for differences between odds ratios. We evaluated interaction as departure of the joint effect from multiplicative and additive null models. Departure from a multiplicative model was assessed using the interaction term in a logistic regression model. Departure from an additive model of interaction was assessed using the relative excess risk due to interaction (RERI).[23] A bootstrap procedure[24] generated 95% bias-corrected confidence intervals of the RERI.[25]

RESULTS

We genotyped 203 subjects with spina bifida and 583 control-subjects for the 844ins68 allele of CBS and for the 677C-T allele of MTHFR. Of the control-subjects, 8.9% ($N = 52$) had one 844ins68 allele. Of the case-subjects, 9.4% ($N = 19$) had one 844ins68 allele. No case or control subject had two 844ins68 alleles. Of the controls, 16.6% ($N = 97$) had two 677C-T alleles, 53.7% ($N = 313$) had one 677C-T allele, and 29.7% ($N = 173$) had none. The corresponding distribution among case-subjects was 25.6% ($N = 52$), 43.8% ($N = 89$), and 30.6% ($N = 62$), respectively. These proportions were similar between subjects born in the North and in the South. Genotype frequencies were similar in the two sets of controls (newborns and adults)[17]; therefore, we pooled the sets.

The odds ratio for spina bifida associated with the joint genotype (homozygosity for 677C-T, heterozygosity for 844ins68) was 3.69, compared with 1.57 and 0.83 for 677C-T homozygosity alone and 844ins68 heterozygosity alone, respectively (Table 1). Adjusting for place of birth and sex did not change appreciably these results. Similar results were obtained when we analyzed separately lipomeningocele and meningocele.

When we assessed the additive interaction we found that the RERI, which estimates the amount by which the joint effect exceeds the sum of individual effects, was 2.30 (95% confidence interval [CI], -0.45–14.02). In the absence of additive interaction, the RERI would be expected to be 0. The odds ratio for multiplicative interaction, which estimates the ratio between joint effect and the product of individual effects, was

Table 1
Estimated relative risk for spina bifida associated with the 677C-T allele of MTHFR and the 844Ins68 allele of the CBS gene, alone or in combination, Italy, 2001

MTHFR[a]	CBS[b]	Cases	Controls	Odds ratio	95% CI	AF-gen[c] (%)	AF-Pop[d] (%)	Genotype frequency in controls (%)
+	+	7	6	3.7	1.0–13.5	72.9	2.5	1.0
+	−	45	91	1.6	1.0–2.4	36.1	8.0	15.6
−	+	12	46	0.8	0.4–1.6	−0.17		7.9
−	−	139	440	Ref				75.5
Total		203	583					

[a]MTHFR: +, two 677C-T alleles of MTHFR; −, none or one 677C-T allele.
[b]CBS: +, one 844ins68 allele; −, no 844ins68 allele.
[c]AF-gen (%): attributable fraction (percent) among cases with genotype.
[d]AF- Pop (%): attributable fraction (percent) among all cases in the population.

2.87 (95% CI, 0.76–10.75). In the absence of multiplicative interaction, such odds ratio would be expected to be 1. We compared our findings with those from the literature (Table 2), rearranging the original data into the two-by-four table design.

DISCUSSION

The findings of this study suggest that interactions between common alleles of folate-related genes may contribute to the risk for spina bifida. Such interactions might be common. Of the control-subjects in this study, for example, 1 in 6 was homozygous for 677C-T allele of MTHFR, 1 in 11 had the 844ins68 allele of CBS, and 1 in 97 had the joint genotype.

The results should be interpreted in light of the limitations of the study. First, the case group included prevalent rather than incident cases; did not include affected pregnancies that were electively terminated, that spontaneously aborted, or that ended in fetal death; and represented an unknown proportion of eligible cases. Such limitations might lead to distorted estimates if entry into the study was associated with genotype. For example, if the allelic variants affect survival, our results may be biased.[26] Some studies have suggested that the 677C-T allele frequency may be lower in older people.[27,28] However, such a lower allele frequency was found among people 80 years of age or older. Another possible limitation is the extent to which the control group is representative of the underlying population. The use of a subset of controls derived from newborn screening programs, the similarity of the genotype frequencies regardless of control subset, place of birth, age, and sex, and the consistency with independent estimates of allele frequency in Italian populations[29–38] suggest that selection bias in the control group, if present, might be small. Moreover, bias is an unlikely explanation for the supra-multiplicative interaction, which is the main finding of the study. In fact, such interactions can be examined even without controls, assuming that alleles distribute randomly in the population.[39] Finally, we do not measure blood folate and, thus, could not investigate its role in disease risk.

The biologic basis of the interaction between allelic variants of MTHFR and CBS is still unclear and awaits further biochemical and genetic studies. Both genes are involved in the metabolism of folate and its associated one-carbon transfers: MTHFR helps maintain an inflow of reduced methyl donors, used during synthesis of nucleotides and methylation of many substrates, including homocysteine[40]; CBS, which is expressed early in embryogenesis in the brain and neural crest,[41] controls one of the outflow paths of homocysteine. Homozygosity for the 677C-T allele of MTHFR has been associated with an increased risk for spina bifida in several, though not all, studies.[9] The role of 844ins68 allele of CBS is unclear. The 844ins68 allele has been reported to cause incomplete mRNA transcription compared with the common allele.[19] This allele, however, unlike the 677C-T allele, has been associated with lower, rather than higher, plasma homocysteine levels compared with the wild-type allele.[42,43] The metabolic and clinical effects of the joint genotype (677C-T homozygosity and 844ins68 heterozygosity) are also inconsistent across studies. For example, the joint genotype has been associated with increased plasma homocysteine levels in some studies,[44] whereas in others the 844ins68 allele has been reported to counter the homocysteine-raising effect of the 677C-T allele.[45] Clinically, the joint genotype has been associated with an increased risk for arterial and venous occlusive disease.[46]

As for spina bifida, our findings and those reported by Ramsbottom and associates[11] (Table 2), in our view, reflect similar patterns of risk, i.e., no increased risk with the 844ins68 allele alone (odds ratio of 0.8 in both studies), an increased risk with 677C-T homozygosity alone (odds ratios of 1.6 and 2.0), and a further, disproportionate increased risk with the joint genotype (odds ratios of 3.7 and 5.1). A third study from Germany,[14] however, reports neither an effect of 677C-T homozygosity nor an effect of the interaction with the 844ins68 allele (Table 2). A fourth study from the United States[15] reports two sets of findings, using different sets of controls, one consistent with no interaction and one consistent with significant interaction (because these researchers did not report the full genotype distribution, we were unable to summarize their data in Table 2). These same investigators also had previously reported no effect of the 677C-T allele on spina bifida risk.[47] It should be noted, therefore, that on balance, studies report ei-

Table 2
Independent and joint effects of 677C-T and 844ins68 in three studies

		This study				Ramsbottom et al.[11]				Richter et al.[14]			
MTHFR[a]	CBS[b]	Cases	Controls	Odds ratio	95% CI[c]	Cases	Controls	Odds ratio	95% CI[c]	Cases	Controls	Odds ratio	95% CI[c]
+	+	7	6	3.7	1.0–13.5	7	5	5.1	1.4–20.9	2	2	1.2	0.1–17.0
+	−	45	91	1.6	1.0–2.4	19	34	2.0	1.0–3.9	26	25	1.3	0.7–2.4
−	+	12	46	0.8	0.4–1.6	16	76	0.8	0.4–1.4	20	40	0.6	0.3–1.1
−	−	139	440	Ref		86	315	Ref		136	166	Ref	
Total		203	583			128	430			184	233		

[a]MTHFR: +, two C677T alleles of MTHFR; −, no or one C677T allele.
[b]CBS: +, one 844ins68 allele of CBS; −, no 844ins68 allele.
[c]95% CI, Fisher exact.

ther both effects—of 677C-T homozygosity alone and the interaction with 844ins68—or neither.

The practical impact of MTHFR–CBS interactions, assuming they are real, can be inferred from their estimated attributable fractions (Table 1). The attributable fraction among those with the genotype (AF-Gen), for example, shows that, even among cases with the genotype, the latter might not be causal. The attributable fraction in the population (AF-pop) shows that approximately 1 in 40 cases in this population (2.5) was due to the interaction, assuming causality. Such proportion might be different in other populations with different prevalences of the 677C-T and 844ins68 alleles. Such estimates, if replicated across other studies and populations, can also help understand better why folic acid works in preventing spina bifida and to what degree genetic susceptibility is involved in different populations.

In summary, our findings support a possible interaction of common alleles in the risk for spina bifida, at least in this population from Italy. We suggest that the 844ins68 allele of CBS be included in studies of putative genetic determinants of spina bifida, in an effort to delineate the role of complex genotypes in disease risk. Because of the ethnic and geographic variation of the frequency of 677C-T allele[9] and possibly also of the 844ins68 allele,[11,19,48,49] it is possible that failure to consider interacting genes may contribute to the variability of the risk estimates.

A further methodologic consideration relates to the impact of sample size on the power to detect interactions. All studies to date (Table 2) have generated effect estimates that are relatively imprecise, with wide confidence intervals. Thus one must avoid the twin pitfalls of believing an effect without considering its potential random variation or dismissing findings as negative solely on the basis of a *P* value. Convincing and precise answers on the presence or absence of interaction awaits evidence from larger, carefully conducted studies.

Ideally, such studies also should evaluate interactions with environmental factors, such as blood folate levels[50] and multivitamin supplement use.[51] Other gene–environment[52] and gene–gene interactions[52,53] might also occur. If feasible, interactions between maternal and fetal genotype should also be investigated. For example, it has been suggested that, although the fetal genotype might play the preeminent role, the coexistence of the 677C-T homozygote genotype in the mother might further stress the fetus, perhaps by exposing it to suboptimal folate levels and, thus, increase the fetus's risk for spina bifida.[54] Ultimately, the prevailing hope is that the study of how interactions between multiple genes and environmental factors contribute to health and disease may generate further opportunities for primary prevention, particularly as relates to relatively common conditions in genetically diverse populations.

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